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EXTERNAL AND INTRINSIC SIGNATURES IN HUMAN TEETH TO ASSIST FORENSIC IDENTIFICATION WORK

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"We are drowning in information, while starving for wisdom. The world henceforth will be run by synthesizers, people able to put together the right information at the right time, think critically about it, and make important choices wisely."
(E.O. Wilson)

ABSTRACT

In forensic medicine, dead victim identification constitutes an important task for forensic professionals including forensic pathologists, anthropologists, and odontologists.

If no clues are at hand regarding the identity of the deceased, whether it is a victim of a mass disaster or a suspect homicide case, it is vital to know when a person died, and to know the sex and age of the decedent in order to limit the search for possible matching persons.

In paper I, teeth from Swedish individuals were examined using both ^{14}C analysis and aspartic acid racemization. The ^{14}C analysis takes advantage of the so-called bomb-pulse, a tremendous increase of ^{14}C in the atmosphere due to thousands of test detonations of nuclear weapons 1955-1963, which allows for an accurate birth dating of modern biological material. The aspartic acid racemization method gives an estimate of the age at death. The methods showed a significant correlation, and by combining them, we showed how both the year of birth and year of death of an unknown skeleton could be determined. In this study, we also found that ^{14}C levels in tooth enamel from Swedish teeth predicted the true date of birth with an average absolute error of 1.3 ± 0.9 years and that analysis of whole crown offered fairly good precision too.

In paper II, the possibility of geographical differences in precision due to uneven distribution of bomb-pulse radiocarbon during the test bomb period was addressed. Interestingly, the ^{14}C determinations predicted the true date of birth with a similar precision even when analyzing teeth from different continents. Conversely, the levels of the stable isotope ^{13}C showed significant difference depending on geographical origin.

In paper III, teeth were collected from North America to find out if differences in stable isotope concentrations can be detected in the teeth from subjects raised in such a limited geographical region. Teeth collected from subjects raised in Mexico showed extremely high ^{13}C values, most likely due to a high consumption of corn and sugar cane. ^{13}C levels in tooth roots were also higher in Mexican subjects compared with persons raised in United States and Canada, but the difference was not as conspicuous. Incorporation of ^{18}O, another stable isotope, is mainly dependent on the drinking water. Analysis of ^{18}O in tooth roots from subjects raised in Northwestern America showed the lowest levels, whereas this marker was not reliable for discriminating between Mexican and southern United States subjects. The ^{14}C determinations of date of birth on North American teeth showed only slightly higher imprecision (average absolute error 1.8 ± 1.3 years) than Scandinavian teeth. In paper III, these and previous tooth ^{14}C. Finally, a reference guide to birthdating persons using tooth ^{14}C values is provided in paper III.

In summary, these studies describe methods to determine date of birth, date of death, and origin of unknown dead victims, information that is expected to facilitate the identification work.

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II. **Alkass K**, Buchholz BA, Druid H, Spalding KL. Analysis of (14)C and (13)C in teeth provides precise birth dating and clues to geographical origin. Forensic Sci Int. 2011;209(1-3):34-41.

III. **Alkass K**, Motani H, Buchholz BA, Holmlund G, Senn DR, Spalding KL, Druid H. Analysis of radiocarbon, stable isotopes and DNA in teeth to facilitate identification of unknown dead victims. Manuscript

Publications not included in this thesis:

I. Prunell GF, Svendgaard NA, **Alkass** K, Mathiesen T. Delayed cell death to acute cerebral blood flow changes following subarachnoid hemorrhage in the brain. J Neurosurg. 2005;102(6):1046-54

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1 INTRODUCTION

1.1 FORENSIC MEDICINE INVESTIGATIONS

The word “forensics” originates from the Latin “forum” meaning square. During the time of Romans, a criminal charge against a person was held before a group of public individuals in the forum (on a square). Both the suspect of a crime and the accusing party would give speeches and explain their version of the event. The individual that could present the most convincing evidence, i.e. with the best forensic skills, would win the case.

Even though Imhotep in Egypt (approx. 2980-2900 BC) is considered to have been the first doctor of forensic medicine, the development of the discipline and the practical forensic medicine casework was not established in the Western countries until the 18th century. For a long time development was slow, but accelerated immensely when DNA techniques were applied in the casework. Today, virtually all countries have forensic experts that assist the legal system to reach fair decisions in courts dealing with violent crimes. In Sweden, the National Board of Forensic Medicine is responsible for all forensic casework except crime lab analyses. This is in contrast to the Medical Examiner and coroner systems that is used in North America, U.K. and in former U.K. colonies. In most European countries, forensic medicine departments are run by universities and typically each of them has their own toxicology, histology and genetics lab, and a forensic odontologist.

1.2 FORENSIC IDENTIFICATION CASEWORK

Regardless of the organization of the activities, identification of unknown dead bodies is a frequent and important task for the forensic medicine departments worldwide. Mass disasters are seemingly becoming more frequent due to the increase in world population and the development of terrorism during the last decades. There are also experts that claim that meteorological statistics support the notion that severe weather phenomena have become more common. Other experts claim there has been no increase, but rather that the reporting has been more intense. However, due to the increase in population including in areas susceptible to e.g. floods, any such event is likely to kill more people. Mass disasters may be due to earthquakes, floods, fires or explosions and will imply substantial efforts to recover and identify the remains of the victims, both for humanitarian and legal reasons. Recent examples of casualties that

have struck Swedes are the sinking of the Estonia ferry on 28 September 1994, which claimed 852 lives (501 Swedes), the Gothenburg discotheque fire on October 30, 1998, in which 63 teenagers and young adults died and the South East Asia Tsunami on December 26, 2004 with a death toll of about 230,000 people (543 Swedes).

Such casualties imply extreme economic costs and individual suffering that are difficult to imagine for those not involved. Identification work will go on for a long time, and many victims may remain unidentified due to incomplete information about missing persons. In addition to such disasters, unknown dead bodies are found on a daily basis worldwide, and these victims also need to be identified, regardless of the cause and manner of death. In the Doe network database (http://www.doenetwork.org), which is extensively used in North America, approx. 3900 individuals were registered as of April 2011. Each year a number of unknown dead bodies registered are identified, but more new cases are added, so the annual number is constantly increasing.

A review of this database disclosed that the estimated age for adult individuals showed an average range of 15 ± 12 years. Such a wide range is not very helpful in the efforts to limit the search for possible matches. Further, the sex was unknown in a considerable number of cases, and the origin of the person rarely known, apart from the information about the place where the victim was found. Hence, a more detailed characterization of the subjects in terms of age, sex and geographical origin should be expected to improve the success rate in dead victim identification work. The different means of identification dead persons are outlined below.

1.3 VISUAL RECOGNITION

Dead bodies that are intact and well preserved can typically be recognized by relatives friends or doctors and health care staff who have treated the deceased. Also, a doctor who certifies the death, or the police, may in these situations be able to make a reliable identification by comparing a recent passport photo or other ID document. Subjects who die in their home may also sometimes be considered to be identical with the resident provided there are no reasons to believe that another person entered the apartment or house and happened to die there while the resident was gone. Hence, the efforts to identify deceased rely much upon the circumstances, and in many instances the police will feel comfortable with very little supporting evidence other than the general appearance of the body and the findings at the scene.

1.4 PERSONAL EFFECTS

Personal effects such as clothing, jewelry, contents in pockets, identification cards, credit cards or membership cards, and various other belongings of the victims may sometimes be considered sufficient for a positive identification. Again, the grounds for the identification may vary depending on the circumstances surrounding death, the place of death and other findings at the scene.

1.5 EXTERNAL EXAMINATION OF THE BODY

In mass disasters, the victims are often not subjected to an autopsy because of the large numbers to be examined. However, typically, an external examination is performed. This will include examination of the skin surface and external orifices of the body, occasional smaller incisions and collection of blood (when available), bone samples and sometimes samples from soft tissues. Upon such an examination, scars from surgery or previous injuries may support or exclude a particular possible matching person. Implants, such as pacemaker or insulin pump may also be helpful. Tattoos, that over the past decades have become more elaborate, can sometimes be sufficient for a positive identification. Further, the length and weight of the body, and the color of the skin, eyes and hair are easy to determine if the body is not severely decomposed. and constitute helpful body characteristics that can be compared with ante-mortem information.

1.6 AUTOPSY

Having stated that autopsy is not always performed on victims of mass disasters, this might be the case in certain such events, e.g. to determine whether a particular death actually was due to the event or unrelated. Also, an autopsy may be necessary to help in the investigation of e.g. a terrorist attack, a multiple shooting attack or an unexpected fire resulting in a limited number of dead victims. Then an autopsy, disclosing the injury pattern, the bullet entry and bullet path in the body or stab wounds can be carefully documented to allow for a reconstruction of the event. This might then help identify one or several responsible assailants. At the autopsy, tissues for DNA-identification can be collected, as well as dental radiographs. The internal examination may also reveal specific pathology, such as previous myocardial infarctions, previous intra- and extracerbral hemorrhages, renal disease, liver cirrhosis, and more. Such information can also be compared with ante-mortem information about a possible

matching person. Today, computed tomography (CT) and magnetic resonance imaging (MRI) are increasingly used in the routine casework and can supplement autopsy findings, e.g. to give a three-dimensional picture of injuries sustained, and disclose missing organs from surgery (such as appendix, gastric reduction, and hysterectomy) and old fractures of the bones of the extremities, which otherwise imply time-consuming dissection procedures.

1.7 FINGERPRINTS

Fingerprints are unique to every person. If fingertips of a dead body are well preserved, representative fingerprints can typically be obtained. In decomposed bodies, the epidermis is sometimes desquamated, but useful fingerprints can in many instances still be obtained. Postmortem dehydration is a more problematic situation. Then, injection of methanol may be tried to restore the original rounding of the fingertip and allow for a fair print. For such subjects whose fingerprints are already in criminal registers, the identification is usually straightforward, but in other cases, representative fingerprints from personal belongings or from the place of living are needed for a positive identification. Dismembered bodies pose special problems, including identification. In such instances, fingerprints may give some clues, since there are certain differences between sexes in the pattern of the dental ridges (1).

1.8 DNA IDENTIFICATION

Certain human remains will be suitable for the traditional identification approaches, particularly if there is substantial fragmentation of the remains. The DNA profiles from recovered mass disaster remains may be compared with the DNA profiles from reference samples such as known personal effects of the victim or with reference samples (such as blood samples, but today typically mouth swabs) from family members. Comparison with DNA from e.g. tooth brushes and razors have proven not to be ideal since obviously many persons share such items, but when using DNA methods that can identify multiple DNA profiles in a specific sample, such approach is still possible. Archival pathological biopsy or autopsy samples constitute a good reference material, and the problems with formalin fixation and paraffin embedding have largely been overcome. However, such reference samples are often not available or may sometimes not be released due to medico-legal regulations aiming at protecting personal integrity.

DNA comprises all genetic information of a specific cell. It is found in the cell nucleus chromosomes (genomic DNA), but also in the mitochondria (mtDNA) of the cell. DNA test results are today widely accepted as convincing legal proofs in court regarding investigations of paternity and crimes when the DNA profile in traces e.g. at the scene or on a victim is compared with the DNA profile of a suspect. Such DNA profiles are also extensively used in human identification. It is possible to obtain DNA from practically any human tissue, such as blood, oral smear, saliva, bone, tooth, internal organs, hair, semen, urine, among other biological materials. The amount of the DNA varies, however, depending on the tissue, and its condition. The structure most resistant to severe environmental conditions such as incineration, immersion, trauma, mutilation and decomposition and still provide material for analysis, is the tooth. In dentin, present both in the crown and the root of the tooth, DNA will typically be well preserved, although in limited amounts. In forensic investigation, DNA analysis is usually performed by polymerase chain reaction (PCR), a technique that allows amplification of DNA at pre-selected, specific sites, and further analysis of specific short tandem repeat (STR) sequences that have a large inter-individual variation. A DNA-identification of a dead victim always requires a comparison between the DNA profile of a sample from the victim and the profile of a relative. The latter samples may constitute a blood sample, but today it is becoming increasingly common to analyze oral swabs. Alternatively, the reference sample may be an extract from a toothbrush, allegedly used only by the decedent. The sample from the dead body may be blood, bone, teeth, or soft tissues, if available. From victims of mass disasters, a sample from femur is usually collected and analyzed as a successful standard procedure.

1.9 NUCLEAR AND MITOCHONDRIAL DNA

The mtDNA is more abundant since the mitochondria are numerous, and hence the total mtDNA is more easy to isolate in a sample containing a limited number of cells. Further, in hair, mtDNA is the only source of DNA. mtDNA is exclusively inherited from the mother, implying that an mtDNA result will match all children of a biological mother, and the mother herself (2, 3). This means that in an investigation of an unknown dead victim with no ante-mortem reference material, mtDNA in hair from the body may match e.g. mtDNA in hair procured from a hairbrush belonging to the decedent or reference samples from siblings or the mother and allow for a positive identification, provided that relatives of the maternal lineage can be excluded (4-6).

Whenever available, nuclear DNA profile is of course advantageous and the methods today allow for analysis of small samples. A recent study reported successful profiling of as few as 15 spermatozoa using laser capture microdissection coupled with on-chip low volume PCR (7). However, more reproducible profiling is achieved if the amount of DNA is larger. In tooth roots, a small piece will typically contain sufficient amount of nuclear DNA for a full profile.

1.10 FORENSIC ODONTOLOGY

Forensic odontology is the discipline of tooth examinations for various medico-legal purposes. In addition to bite mark analysis and age estimations (of e.g. of immigrants) one of its main applications is in the identification of human remains, particularly in mass disasters where other forms of identification may not be applicable due to mutilation or severe burns. Teeth are the most enduring part of the human body. Although we are all born with the same number and types of teeth, the dental characteristics of each individual are unique. Dental medical records and/or dental radiographs are today widely available for comparison in most Western Countries. Having stated that, in all countries, it may be difficult to find the particular dentist who have treated the person. This is often a time-consuming task for the police and/or for the forensic odontologist.

1.11 DENTAL IDENTIFICATION

Experienced forensic odontologists may be able to recognize fillings that are more or less characteristic of a specific country or of a limited geographical region. This is a good start, should there be no clues to the origin of the subject. However, the dental status of the younger generations, which will be gradually more represented in the forensic casework, is getting better, and an increasing number of young adults will not have any fillings or dental restorations at all. In addition, the increased use of dental braces comprises a challenge to professionals by producing a large number of look-alikes. The basis of odontological identification is that postmortem dental remains can be compared with the ante-mortem dental records, including written notes, study casts and dental radiographs (see Figure 1) to confirm identity. Individuals with numerous and complex dental treatments are typically easier to identify than those with little or no restorative treatment. Features examined in dental identification are teeth present (erupted or not), missing teeth (congenitally, or lost ante-mortem), tooth type (e.g. permanent, deciduous, mixed), tooth position, crown morphology (size and shape,

enamel thickness, contact points), crown pathology (caries, attrition abrasion), root morphology (size, shape number), pulp chamber/root canal morphology (size, shape and number and secondary dentine), dental fillings (metallic, plastic composite), dental implants, bridges, partial and full removable prosthesis (for review see (8)). All these features may be listed in a table or registered in a form with a dentition chart, and compared with the characteristics of a missing person, but the forensic odontology identification work almost exclusively also include a careful visual comparison of the detailed tooth morphology (often including an in-depth analysis of the fillings and other restorative treatments) with ante-mortem radiographs or other dental records if radiographs are not available.

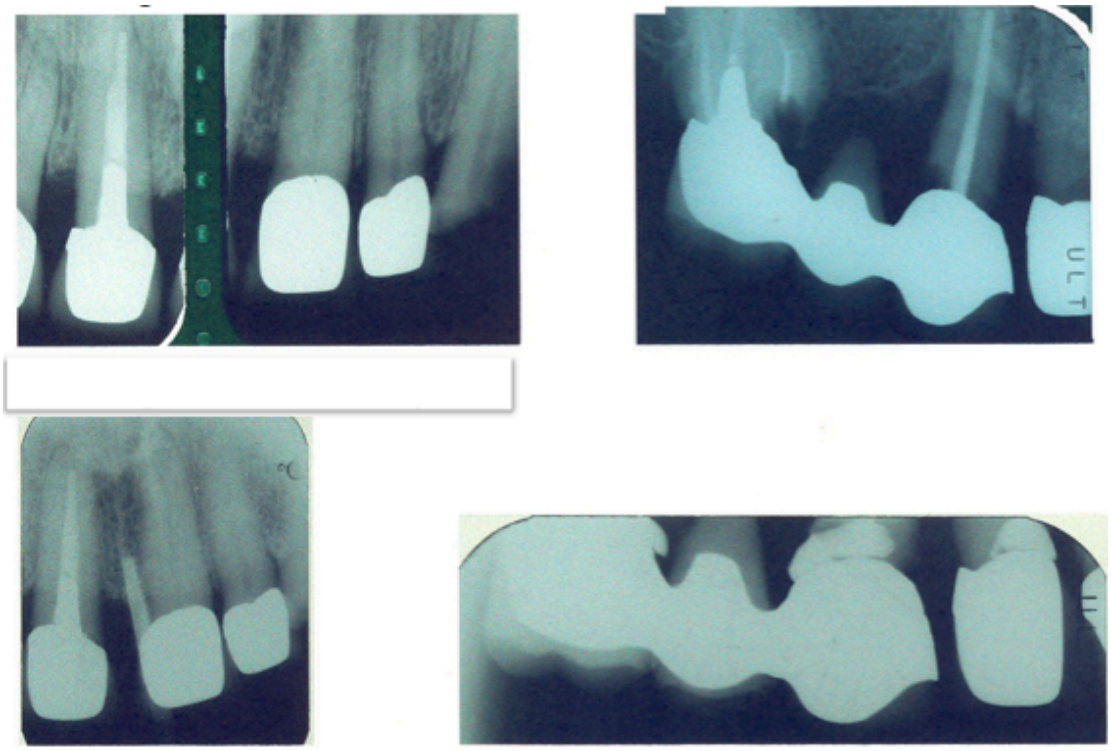

Figure 1. An example of ante-mortem and post-mortem radiographs showing several characteristics supporting a positive identification.

1.12 AGE ESTIMATION

Age determination of unknown human bodies is important in the setting of a crime investigation or a mass disaster, because the age at death, birth date, and year of death, as well as gender, can guide investigators to focus on suspect perpetrators (and/or victims) among a large number of possible matches. We reviewed the Doe Network Database (http://www.doenetwork.org), and found that 885 out of 1188 cases were estimated to be 20-50 at the time of death (=75%). This means that almost all dead

bodies found today (provided that most of them died within the last decade) will have bomb-pulse radiocarbon incorporated in the enamel of their permanent teeth, allowing for an analysis of the ^{14}C level to more closely estimate the their year of birth. Anthropological examinations often give an estimate of the age of the victim with an SD of 15 years, which is not very helpful when investigators want to select possible matches within e.g. an age range of 25-45 years. Such estimates will match the age of a large number of persons whereas a birthdating of the individual with a high precision, e.g. with bomb-pulse carbon dating, will limit the number of possible matches to perhaps 10% or less. The figures in the Doe Network Database may not be representative for the best anthropological methods, which may be due to either the use of less accurate methods, more conservative estimations, or due to lack of appropriate material.

In some situations, the age estimation of a skeleton without teeth may be performed using examination of the cartilage-bone junction of the ribs (9, 10). This can either be examined by the naked eye, by microscopy or by x-ray examination. The later allows for a differentiation of specific stages that can give a fair age estimation of the age at death. The same applies to the radiologic appearance of the pubic symphysis, first reported by Brooks et al (11) and later further developed to assist in both age and sex determination (12), se Figure 2.

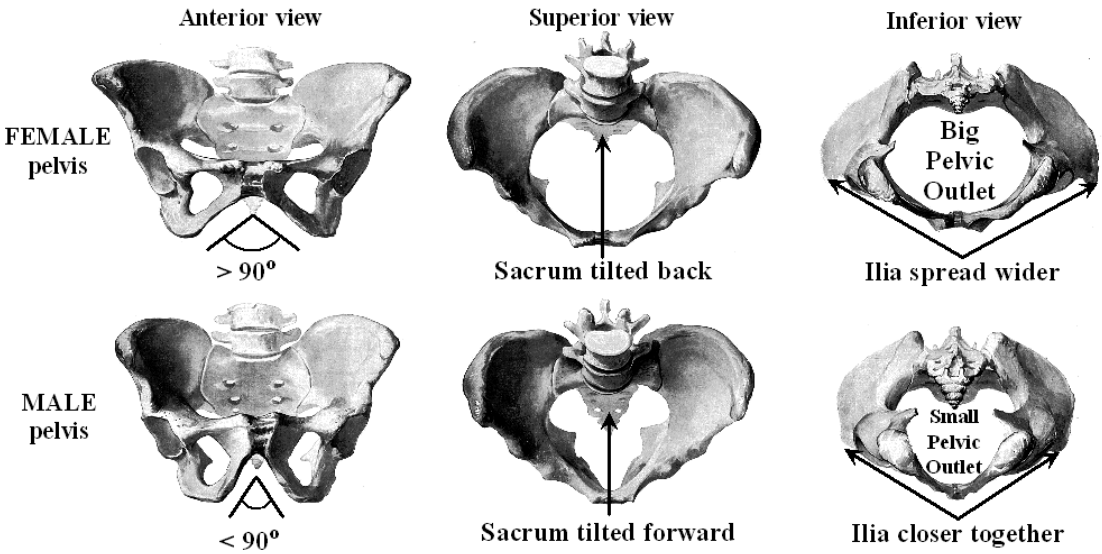

Figure 2. Typical morphological differences between the male and female pelvis.

Both these methods seem to become less reliable with older actual age, and in subjects older than 50 years, they tend to be less useful.

Further, a comparative analysis may be performed using the skull sutures, the zigzag seams where the bones of the skull meet. However, the precision of this method is poor, even when a high-resolution CT scan is applied; for example the stage 4 of the sagittal suture closure was reported to be at minimum 27.56 years and at maximum 88.19 years (13). The authors conclude that endocranial sutures (inside the skull) are more reliable as an aging method than ectocranial sutures. Still, the images of skull suture closure obviously show a very large time frame, making this approach less useful as a guidance to the police or identification teams in the efforts to limit the search for possible matching missing persons.

1.13 TOOTH DEVELOPMENT

Tooth development (odontogenesis) constitutes a long and complex process that is directed by the sonic hedgehog homolog (SHH), a protein which is crucial for organogenesis in vertebrates. The whole process encompasses the early proliferation of embryonic cells to the formation of the hydroxyapatite of the enamel and the production of the dentin and cementum, in which more organic components are trapped when completed.

There are several methods that can be used to determine dental development. Most of these are dependent on the degree of tooth mineralization, which can be observed on dental radiographs. The tooth development, including the eruption, has different time frames for each type of tooth and reference information about these time frames is used to estimate the age of the individual.

The tables of Schour and Massler published in 1941 are a classic example of an atlas approach (14), se Figure 3. They described about 20 chronological stages of dental development starting from 4 months after birth until 21 years of age. These tables have been extensively used in age determination of children for various medico-legal purposes. Demirjian et al. tried to simplify chronological age estimation and restricted the number of stages of tooth development to 8 giving them score of “A” through “H” and confined the analysis to the first seven teeth of the left lower quadrant, i.e. mandibular teeth (15). They used statistical methods to assign a maturity score for each of these seven teeth to almost each of the 8 developmental stages and reported results separately for boys and girls. By combining these scores for the teeth of the left lower

quadrant a sum value was obtained for different ages, resulting in a reference table to be used in the estimation of chronological age.

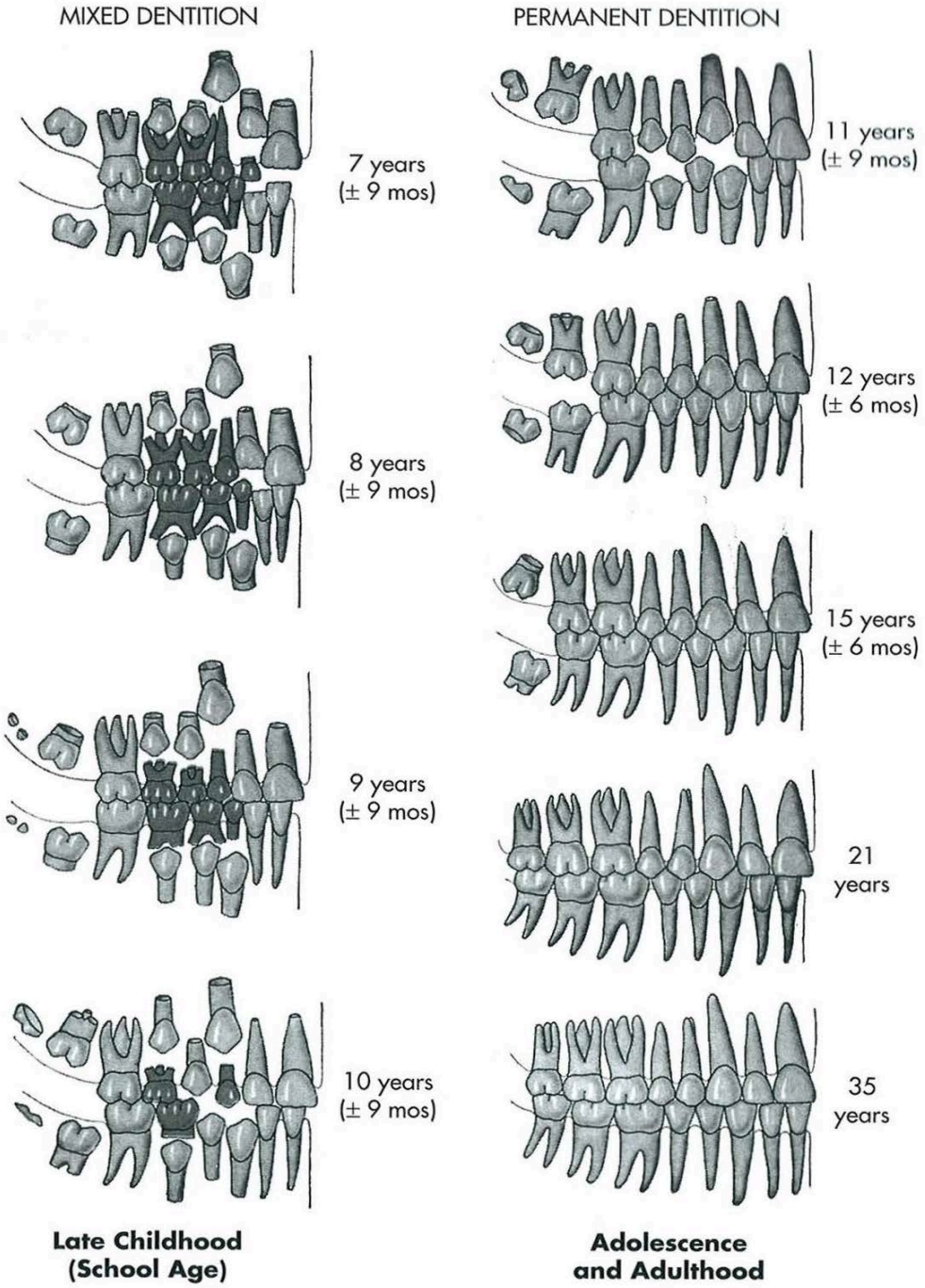

Figure 3. Development of the human dentition from the seventh year to maturity. (From Schour L, Massler M: The development of the human dentition, *J Am Assoc* 28: 1153, 1941).

For several teeth there are differences in the time frames of tooth development between sexes (14, 16, 17) implying that an age estimation of a skeleton will be somewhat less accurate if the sex is unknown. There are many publications reporting

on age estimation of individuals from observations of tooth development. A high degree of accuracy is achieved by determining the mineralization stage of each tooth or each tooth category as described by several authors (15, 17, 18) The basis of Demirjan´s method (15) is to determine the onset of mineralization until the completion of root formation. This method defines eight mineralization stages for premolars and molars and six steps for incisors and canines of mandibular teeth and has been widely cited and used in routine casework. However, since the method basically stipulates stage determination of several teeth, missing teeth in the left lower quadrant will reduce the precision.

According to Hägg and Matsson (19), precision was found to be high, especially in younger age groups, and more precise than the method of suggested by Liliequist and Lundberg (18). For our studies, we used Nolla´s method, which defines ten developmental stages of each tooth (17). Nolla´s results are based on approximately 70 dental radiographs of 25 boys and 25 girls of caucasian origin - a study that can hardly be repeated because of the concern about the possible medical hazards with radiological examinations. The precision of the age estimation has been unsurpassed despite further efforts by many investigators, since the use of repeated dental radiographs of children have not been possible.

A study by Haavikko (20) demonstrated no difference in tooth development between Finish, i.e. European children, and Caucasian children in the United States. These results are important for the ^{14}C analysis of teeth when Nolla´s references for enamel formation is used. However, a study by Holtgrave *et al.* (2007) showed that a slight, but significant acceleration of tooth development had occurred during the past decades in boys, possibly because of improved general nutritional status (21). In contrast, a recent study reported an underestimation of actual age using both Nolla´s and Haavikko´s reference data for both boys and girls, whereas Demirjian´s method produced an overestimation of actual age (22). A previous study by Kahl and Schwarze (16) found that mineralization had rather been retarded today in comparison with tables published by Schour and MassIer in 1941. These seemingly conflicting results may be explained by the examination of dental radiographs of different geographical populations, and systematic inter-observer differences when determining the different tooth developmental stages. However, the differences are still not very large, and hence, the use of any of these references to estimate the age of a child, will have a precision that is useful for most medico-legal purposes.

During repeated radiographic examinations of the same children covering most of the dentition period, Nolla (17) reported the average age for reaching each of the stages

she defined for the different types of teeth. These reference data can be translated into a table showing the average age for stage 4.5, which corresponds to the mean formation time of each tooth (Table 1). The average formation time for the root is believed to be 8.5, and the corresponding times are displayed in the same table.

Table 1. Formation times of each type of tooth according to Nolla (17), based on stages 4.5 and 8.5.

Tooth number		Enamel laydown time (years)	
		Girls	Boys
11	21	3.2	3.2
12	22	3.7	4
13	23	3.8	4.7
14	24	4.9	5.6
15	25	5.6	6.6
16	26	3	3.3
17	27	5.8	6.5
18	28	11.2	12.6
31	41	2.5	2.5
32	42	2.8	3
33	43	4.1	4.3
34	44	4.4	5.1
35	45	5.7	6.5
36	46	2.3	2.4
37	47	5.6	6.5
38	48	11.8	13

Tooth number		Root laydown time (years)	
		Girls	Boys
11	21	8.3	7.4
12	22	8.1	8
13	23	9	10.2
14	24	9.7	10.9
15	25	10.7	11.7
16	26	6.8	7.4
17	27	11	12.4
18	28	16.7	>17
31	41	6	6.6
32	42	6.5	7
33	43	8.7	9.8
34	44	9.3	10.6
35	45	10.8	11.3
36	46	6.7	7.2
37	47	11.8	12.2
38	48	>17	>17

For adult subjects, age estimation from dental radiographs is more difficult, since teeth do not change their appearance much during adulthood. Having stated that, there are certain changes that gradually occur during aging. There are many publications regarding age estimation based on correlations between age and ratios of the height and width of teeth and the pulp chamber as determined from dental radiographs (23-25)

In a recent report, Aboshi *et al.* concluded that pulp–tooth volume ratio is an age-dependent variable which can be used to estimate age with reasonable accuracy (26). They reported an R^2 value of 0.698 for lower second premolars using microfocus X-ray computed tomography. The precision for this and similar morphometric methods is however different in various age groups, and the age-related changes apparently differ between sexes and between different populations.

Gustafson described a technique based on the measurement of regressive changes in teeth by combining six factors seen in tooth sections such as the amount of 1) occlusal

attrition, the amount of 2) coronal secondary dentine formation, 3) the loss of periodontal attachment, 4) the apposition of cementum at the root apex, 5) the amount of apical resorption and 6) transparency of the root (27). Such an approach may be applied on extracted teeth only, and hence for age estimation of living subjects, either physical examination or radiographic techniques must be used.

1.14 ASPARTIC ACID RACEMIZATION

Amino acid racemization is used to determine relative dates of biological materials such as bone, shell and teeth and has been used in archaeological studies for some decades. The method is based on the observation that amino acids can exist in two different forms: levorotatory (L) and dextrorotatory (D), so-called enantiomers. Due to difference in the three-dimensional structure of the enantiomers, a protein containing the D-form of an amino acid may not bind to e.g. an active site on an enzyme. In living organisms the amino acids in protein are almost exclusively the L-form and the D/L ratio therefore close to zero. After death proteins break down and the D and L forms start to interconvert. This process is called racemization. Eventually the D/L ratio approaches one. Hence in the interval between 0 and 1 the D/L ratio is can be used to calculate age of bones, provided that reference data are available. However, the inter-conversion after death is very slow. At a temperature of +25^{o}C it would take approximately 100,000 years for all L-forms of amino acids present in living tissues to undergo complete racemization to the D-amino acid form (28). In contrast, a gradual formation of the D-form of aspartic acid was observed to occur in dentin during life with an average rate of 0.1% per year (29). The analysis of the D/L ratio of aspartic acid in remains of proteins trapped in teeth can therefore be used to estimate the age at death of unknown dead bodies. The conversion after death occurs at a much slower pace, probably partly due to lower ambient temperature. Hence if the postmortem interval does not exceed several decades, the postmortem changes can probably be neglected (30). Since the key publications by Helfman and Bada (28, 29) a large number of studies describing the precision of this method have been published (31-34). These studies are based on the separation of the L-and D-form by gas chromatographic methods. A typical chromatogram is shown in Figure 4. From this figure, it can be conceived that the amount of D-form present in the sample is the critical factor; in young subjects, not much of the L-form has been converted, implying that the method is less accurate for age estimation of young adults, beacuse the amount of D-form may

in some subject be under the limit of quantification(33-36). Aspartic acid racemization analysis for age estimation has been carried out on both tooth enamel and crown dentin. However, analysis of crown dentin was shown to give more accurate age estimation than dental enamel (28, 29). These results have been repeated by several investigators and typically a high correlation between aspartic acid racemization ratio and age has been reported (for review see (35)). In a recent study on Scandinavian teeth from subject across a wide age range we found an overall absolute error of 5.4 +/- 4.2 years (37), but other studies have shown a somewhat higher precision (30, 33, 34).

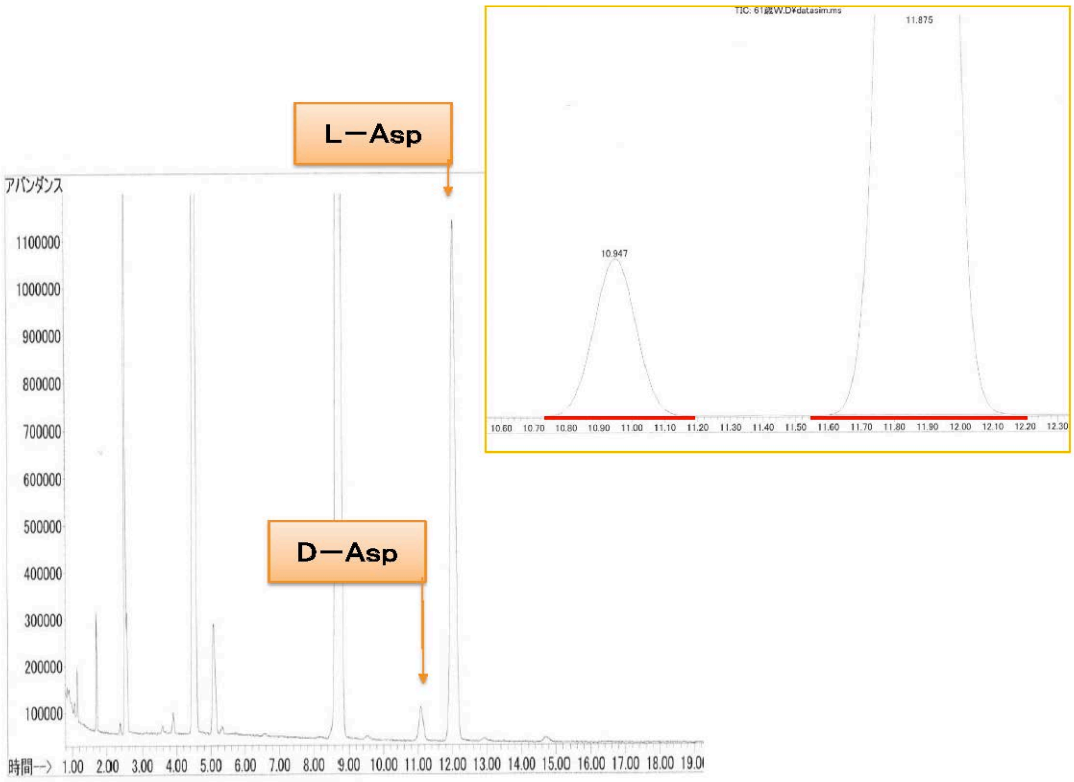

Figure 4. A typical gas chromatogram showing a small peak for the D-form and a huge peak for the L-form of aspartic acid.

1.15 ANALYSIS OF BOMB-PULSE DERIVED 14C

In contrast to aspartic acid racemization analysis and other methods for age estimation, radiocarbon dating of enamel provides information about the date of birth of an individual rather than the age at death. If a tooth is collected from a body that recently died, all methods will estimate the date of birth (although with different precision), but when the examination concerns a tooth from a body that has been dead for several years, only the radiocarbon method will tell the year of birth. This method utilizes

incorporation of so-called bomb pulse derived radiocarbon in our body and is based on the following facts. Radiocarbon, or carbon-14 (^{14}C), is continuously produced naturally in the atmosphere by cosmic ray interactions with nitrogen-14, knocking away one proton from the atom nucleus (Figure 5).

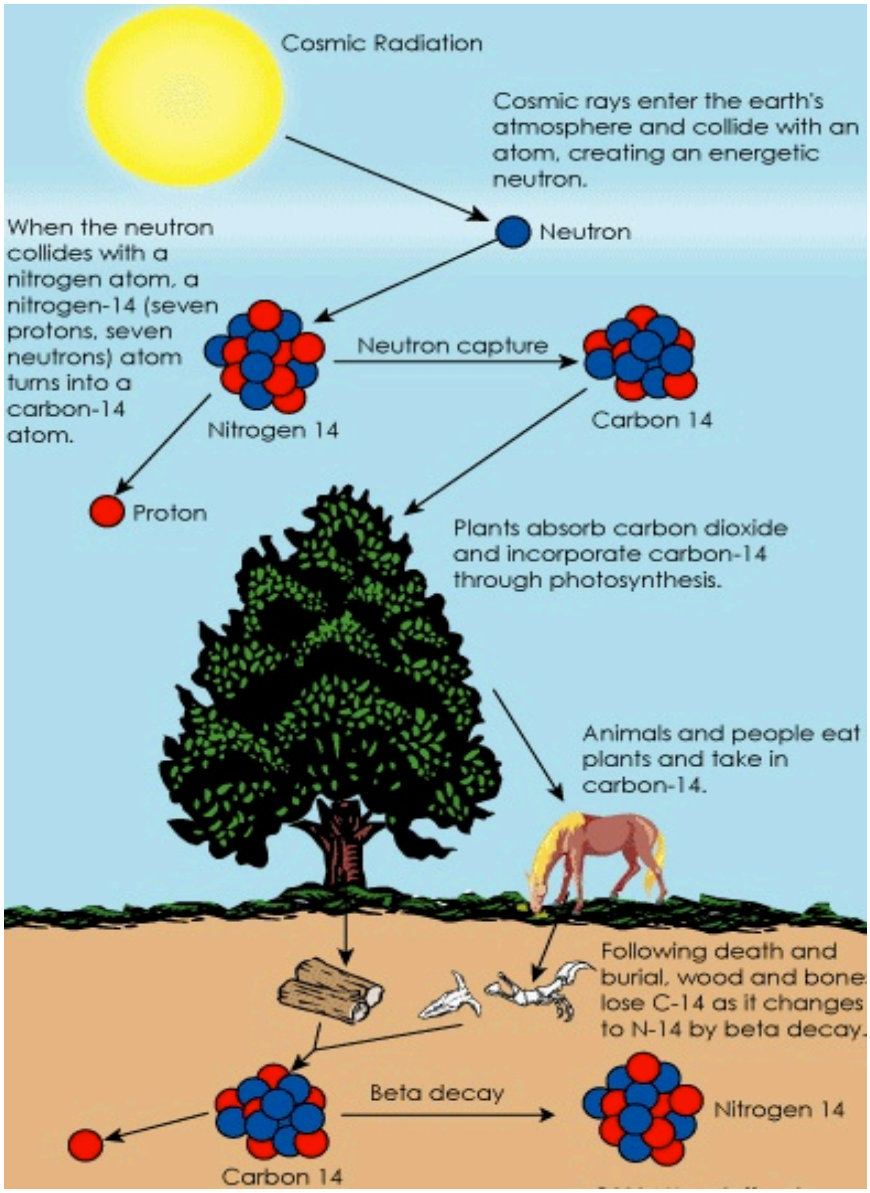

Figure 5. The natural formation of ^{14}C and its further fate in the biosphere.

Isolated carbon atoms in the atmosphere are chemically active and are quickly oxidized to form CO_2. Then CO_2 is incorporated into plants via the process of photosynthesis. By eating plants, and animals that live off plants, the ^{14}C concentration in the human body closely parallels that in the atmosphere at any given point in time (38, 39). Radiocarbon levels in the atmosphere have remained almost constant for several thousands of years before the bomb pulse (40). Only slight variations apparently

chiefly due to small changes in the Earth's magnetic field and the solar cycle have been observed. However, during the cold war (1955-1963) more than 2000 above ground detonations of nuclear weapons were performed, resulting in a doubling in the atmospheric concentrations of ^{14}C (41, 42). Although nuclear weapon testing was carried out at only a few locations, excess levels of ^{14}C in the atmosphere rapidly dispersed and eventually equalized around the globe with a slight lag in the Southern hemisphere. Since 1963, a world-wide test ban treaty was signed, and since then the ^{14}C levels in the atmosphere have decreased exponentially, with a mean atmospheric half-life of 16 years. This should not be mixed up with the radioactive half-life of ^{14}C, which is approximately 5,730 years. Hence, the ^{14}C has not been destroyed or decayed but has moved out of the atmosphere due to mixing with large terrestrial and particularly marine carbon reservoirs. Since radiocarbon is incorporated into all living things, this bomb pulse (see Figure 6) forms an isotopic chronometer of the past 60 years.

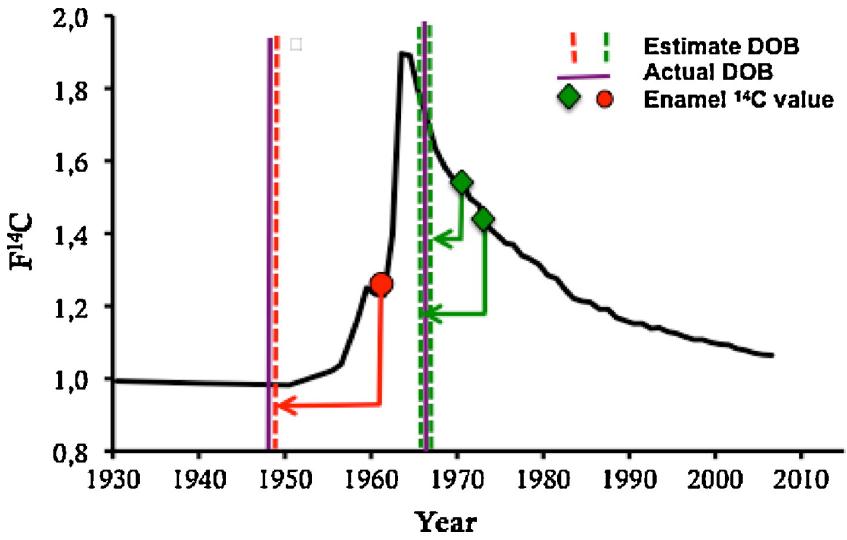

Figure 6. The basic principle behind the birth dating of individuals using radiocarbon levels in tooth enamel. Solid red and green vertical lines are the actual date of birth of two individuals, and shaded lines the estimate, which is calculated by subtracting the average formation time of the particular tooth from the year corresponding to the intercept of the radiocarbon reading. The green diamonds show the ^{14}C values of two teeth with different laydown times, showing that their order of formation only fits the falling part of the bomb curve.

The bomb pulse ^{14}C method should not be mixed up with the Libby method (for which the late professor Willard F. Libby was awarded the Nobel Prize in Chemistry 1960) for age determination of old biological material (43) which employs the radioactive decay of ^{14}C in dead things. During life, the ^{14}C levels will constantly match the atmospheric levels in tissues with a reasonably rapid turnover. When the organism dies, the ^{14}C in the tissues will gradually decay, and with sensitive methods very old material can be dated by comparing the degree of ^{14}C depletion. In oil, no ^{14}C can be found

since it was formed so many thousands year ago that all ^{14}C has been converted back to ^{14}N through beta decay. The increasing use of fossil fuels, starting in the late 19th century resulted in a depression of atmospheric and biospheric ^{14}C levels due to dilution by the ^{14}C-free fossil fuel emission, which also can be appreciated from Figure 5 (slight down-slope from 1930). Hence both methods are based on the measurement of the proportion of ^{14}C in samples, but different references are used to interpret the results. Further, these methods imply an analysis of ^{14}C and ^{12}C with accelerator mass spectrometry (AMS), which should be separated from the analysis of ^{14}C amounts in a sample using a beta-counter. The latter type of analysis requires high concentrations of the isotope, delivered e.g. to experimental animals in radiolabelled tracers. In the atmosphere, ^{14}C is present in extremely small amounts: approximately 0.0000000001 % of the total carbon. ^{14}C constitutes 98.89% and ^{14}C 1.11%. Thus, one carbon-14 atom exists in nature for every 1,000,000,000,000 ^{12}C atoms in living material. It should therefore not come as a surprise that the method to determine the $^{14}C/^{12}C$ ratio needs to be extremely sensitive, and that any contamination with contemporary or nonmatching older material can induce large errors.

Atmospheric carbon concentrations are reflected in the isotopic carbon content of growing plants. New leaves are produced in days to weeks while larger fruit and vegetables form over the period of a month or two. Herbivores lag the atmosphere slightly because their primary carbon source is weeks to months removed from the atmosphere. Omnivores and carnivores lag the atmosphere somewhat more since their carbon sources are another step behind in the food chain. The time of formation of a tissue can be estimated from the bomb-curve by appreciating these lags in incorporation and relating the ^{14}C concentration with the date. Enamel formation can occur over several years in humans so in order to calculate the date of birth of an individual it is important to use reliable data about the average time of enamel formation from each type of tooth. Valuable sources are studies compiling dental radiographs of children of known ages (14, 17, 44). The time of formation for the particular tooth is then subtracted from the radiocarbon date to give the date of birth. The radiocarbon result can cross the bomb curve at two positions, on the rising and the falling part of the curve. If it is obvious that if the person is very old or very young, the appropriate alternative is hardly difficult to select. However, higher values can be more tricky, and in such instances analysis of two teeth with different laydown times may be necessary to tell which of the alternatives that matches the order of their formation.

Of course, other tissues than teeth can be analyzed. Recently, Lynnerup *et al* (45) reported that the lens of the eye could be used as a valuable material for ^{14}C analysis since there is obviously no exchange of carbon between the lens crystallines and the surroundings and hence they showed that the lens ^{14}C levels matched the date of birth of the individual. For subjects lacking teeth, the lens can then serve as a useful alternative for radiocarbon dating (provided that the body is not too decomposed). Human cartilage has a slow turnover, and Libby (38) reported that cartilage from old subjects having been exposed to atmospheric bomb-pulse radiocarbon for almost ten years contained essentially no amounts of bomb-pulse derived radiocarbon. Most other tissues such as bones continuously turnover throughout life, although at different, and often not very well established rates. Whereas most soft tissues are likely to show values reflecting time of death or small delay, bone turnover is somewhat slower. In a forensic context, such as the examination of loose bones, radiocarbon analysis of both trabecular and cortical bone may be helpful, since they obviously have different turnover rates, and hence might help to separate if the values match the rising or falling part of the bomb curve (46). For the determination of the year of birth of an individual, however, none of these types will be suitable due to the turnover, which is also age-dependent. Having stated that, analysis of bones can at least indicate that a person was alive after the onset of the bomb pulse and analysis of several different tissues (if available) with different average turnover times may provide some clues as to the approximate birthdate of the person, provided that more firm reference information can be produced regarding the average lag in radiocarbon exchange for each of them in subjects of different ages.

1.16 STABLE ISOTOPE ANALYSIS

To limit the search for possible matches, the geographical origin of the deceased may also provide clues to the identity. The individual and geographical differences in stable isotopes are explained by variation in the amounts in the diet and in drinking water Atoms of almost all the chemical elements (carbon, oxygen, hydrogen, etc.) have more than one possible atomic weight. For example oxygen (O) has three naturally occurring isotopes, ^{16}O, ^{17}O and ^{18}O. These isotopes do not disintegrate as opposed to the better known radioactive isotopes, which gradually decay at specific rates under the emission of alfa- beta- or gamma-rays.

The relative levels of the stable isotopes of an element are roughly the same regardless of material analyzed. However, thanks to sensitive analytical methods that can separate

and quantify stable isotopes (stable isotope ratio masspectrometry) even small changes in the relative abundances caused by natural processes can be detected. Hence, in birds, analysis of stable isotopes in feathers formed during the winter has been used to determine wintering areas (47, 48). In humans, geographically different stable isotope patterns were observed in hair samples from subjects living on different continents (49). That study is recent and suggests that cultural and/or cultural differences in diet obviously prevail despite the worldwide increase in fast food intake composed of similar basic components. We have recently reported that such geographical differences regarding ^{13}C can be detected in tooth enamel (50). This isotope is incorporated into the tissues of animals, including humans, in relation to the content in food. Since the diet by tradition varies geographically, and is based on different primary products, derived from plants with different ^{13}C content, the amounts in tissues will vary accordingly (51). The reason for the cultural/geographical variation is the different ^{13}C levels in different types of food. Certain plants can discriminate between ^{12}C and ^{13}C, causing differences in the levels of this isotope between different types of plants. So-called C4 plants (which include corn and sugar cane) contain higher levels of ^{13}C than so-called C3 plants (which include potato, wheat and sugar beet). C4 plants are more common in in hotter or drier climates than C3 plants. This in turn means that animals, including humans, having a diet based mainly of C4 plants and/or on animals that chiefly live off C4 plants, will incorporate more ^{13}C than those that have diets based mainly on C3 plants. Another stable isotope that shows geographical variation is the oxygen isotope ^{18}O, but for a different reason. The incorporation of ^{18}O in animal tissues is almost linearly correlated to the concentrations in drinking water, and these concentrations vary with latitude due to differences in evaporation and condensation propensity between ^{16}O and ^{18}O (52). As illustrated in Figure 7, ^{18}O concentrations in tap water in the United States collected 2002-2003 show a gradient with increasing levels from the northwest towards the south and southeast (53). Because of differences in precipitation and variation in ground water supplies to different regions, these levels may change somewhat over time. Still, the ^{18}O levels are not expected to change dramatically over time, and hence the levels in teeth, bones and hair should mirror the levels in tap water at their place of living fairly well.

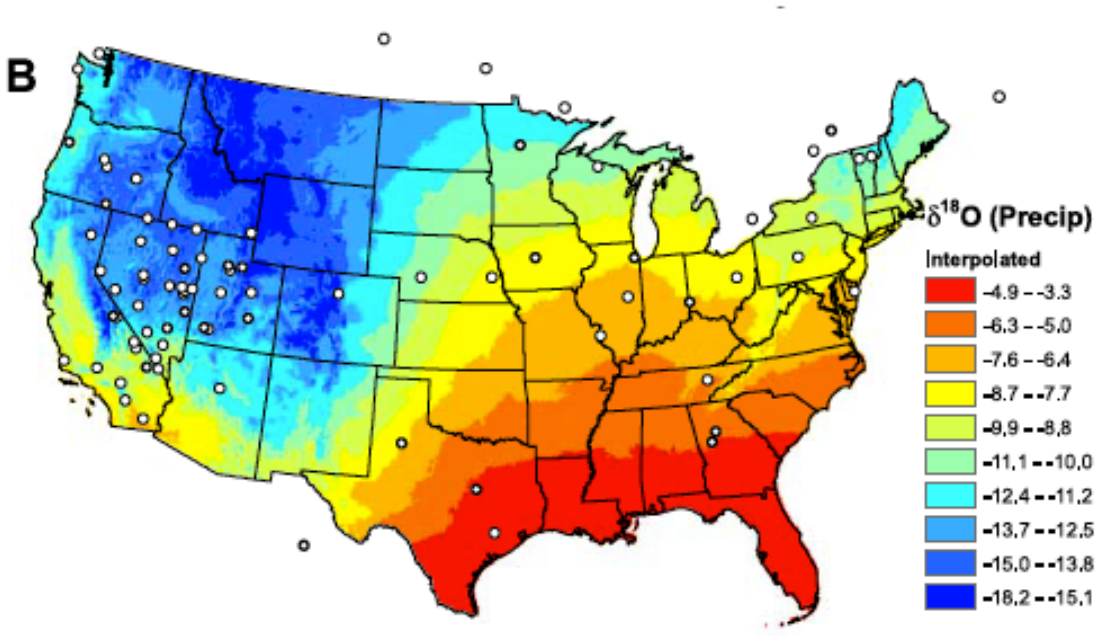

Figure 7. The map shows the ^{18}O levels in tap water across the United States. Please note that the differences also are related to different altitudes.

1.17 AIMS OF THE THESIS

To compare the precision of the bomb-pulse radiocarbon birthdating method of teeth using the standard preparation of teeth with a more rapid approach.

To compare the precision of the ^{14}C method for age determination with the precision of the aspartic acid racemization method.

To use both methods in combination to estimate both year of birth and year of death.

To explore the variation in precision of the ^{14}C method by analysis of teeth from different geographical regions, and during the rising and falling part of the bomb curve.

To explore differences in ^{13}C levels in teeth from different geographical regions.

To compare ^{13}C levels in enamel and tooth roots.

To investigate the possible use of ^{18}O to assist in geographical mapping.

To explore the possibility to analyze DNA in dentin to obtain a individual DNA profile and to determine the sex of the subject, in parallel with ^{14}C and ^{13}C in the same tooth.

2 METHODS

2.1 ANALYSIS OF BOMB-PULSE DERIVED ^{14}C

Enamel Preparation

The standard procedure was as follows. Each tooth crown was cut away from the root at the level of the cervical line and incubated in 10 N NaOH at room temperature in a water bath sonicator (Branson 150). Every 24 h NaOH was replaced, and the non-enamel structures were removed mechanically using an odontologic electric drill. Purified enamel was then washed three times with ddH_2O, then resubmersed in 10 N NaOH, and again sonicated. This procedure was repeated every day for 3–5 days until all dentin and soft structures were removed. Finally, the enamel was rinsed several times in ddH_2O and dried at room temperature overnight. Ten teeth were processed separately by cryo-crashing in liquid nitrogen (see next paragraph), a procedure routinely used for the analysis of bone samples.

Rapid Preparation

The crown of the teeth was separated from the root of the tooth, but the entire crown was washed in ddH_2O and crushed using a liquid nitrogen-filled cryogenic impact grinder (SPEX 6850 Freezer Mill). Using a standard program with a run time of 15 min, this procedure results in a fine powder. Further, one whole tooth crown was washed with ddH_2O and without further preparation subjected to ortophosphoric acid digestion for AMS analysis. All samples were weighed and kept sealed in glass tubes until pretreated for AMS analysis.

AMS Pretreatment

Aliquots of the enamel samples were put in culture tubes for pretreatment to remove the surface carbon that may have contaminated the enamel between formation and analysis. Since the carbon content of enamel is about 0.5%, 80–150-mg aliquots were typically used to get samples containing 0.4–0.9 mg of carbon for ^{14}C analysis. Enamel samples were immersed in 1.0 N HCl at room temperature for 1.5 h, rinsed three times with ddH_2O, and put on a heating block at 95°C to dry overnight.

Powdered samples react vigorously in 1.0 N HCl and were immersed for only a couple of minutes, and were rinsed five times with ddH_2O, and put on a heating block at 95 °C under a loose aluminum foil tent to dry overnight. The acid pretreatment was designed

to carefully remove any possible ^{14}C-containing contamination materials on the outer surface of the enamel without destroying too much of the enamel. Alkaline solutions always contains some carbonate that can potentially exchange with the enamel during the preparation step. Furthermore, alkali solutions remove CO_2 from the atmosphere and produce carbonate and bicarbonate in solution which can aggregate. The dried enamel samples were divided into 5–10 pieces, put in individual single use reactors, and again weighed to the nearest 0.1 mg. The acid etching method dissolves a couple mg of exterior enamel surface in a 100 mg enamel sample. The enamel samples placed in individual reaction chambers were evacuated, heated, and acidified with concentrated orthophosphoric acid at 90°C. The evolved CO_2 was purified, trapped, and reduced to graphite in the presence of an iron catalyst in individual reactors (54, 55). With the enamel aliquots used, almost all CO_2 samples contained >0.5 mg of carbon. If possible, the CO_2 was split, and $\delta^{13}C$ was measured by stable isotope ratio mass spectrometry. Background values were controlled by consistently following procedures, frequently baking sample tubes, periodically cleaning rigs, and maintaining a clean laboratory (56).

AMS Sample Measurement and Analysis

Graphite targets were measured using a 10-MV High Voltage Engineering Europa FN-class tandem electrostatic Accelerator Mass Spectrometry (AMS) system at the Center for Accelerator Mass Spectrometry at the Lawrence Livermore National Laboratory (LLNL), Lawrence, California. The instrumentation procedure is similar to that described by (57) when performing high precision measurements of 18,000-year-old turbidities used as secondary standards. Details on the design of the LLNL AMS system and its operation can be found in the literature (57-60). The system uses an LLNL-designed high output negative ion solid graphite cesium sputter source (58), which emits 250–350 μA of $^{12}C^-$ from a standard sample, corresponding to approximately 900 ^{14}C counts/s from a contemporary sample. The AMS system routinely achieves 15% total system efficiency for carbon (59). Enamel sample analysis run times are rapid, generally less than 5 min. The enamel samples are measured for 30,000 ^{14}C counts/cycle for 4–7 cycle repetitions and give standard deviations of 0.3–0.8%.

A very important ancillary procedure is to compensate for possible background since ^{14}C only makes up 10^{-15} to 10^{-12} of the carbon analyzed. Corrections for such background contamination introduced during AMS sample preparation are performed

by establishing the contributions from contemporary and fossil carbon following generally accepted procedures (40, 61).

The data are normalized using six identically prepared NIST SRM 4990B (Oxalic Acid I) primary standards. NIST SRM 4990C (Oxalic Acid II), IAEA-C6 (62) and Third International Radiocarbon Inter-comparison wood (63) are used as secondary standards, and quality controls, to assess spectrometer performance. The ratio of NIST SRM 4990C to NIST SRM 4990B (Oxalic Acid II/Oxalic Acid I) measured between February 2005 and July 2009 on 22 different sample wheels containing enamel samples showed an average value of 1.291 ± 0.002 (1 S.D.), hence close to the certified value of 1.293 ± 0.001. ^{14}C-free calcite serves as background material for processing the enamel samples. The enamel samples are organized in groups of 10–14 unknowns interspersed between primary standards with one primary standard in the middle of the group. The secondary standards, primary standards, and group of unknown samples are measured consecutively in one cycle. After the completion of a cycle, the set of primary standards, secondary standards, and unknown samples are measured again until acceptable precision is reached. A typical group of 14 enamel samples will be measured in 2–3 h. The measurement error is established for each sample and generally ranges between ± 0.2 and 0.8 % (1 S.D.). All ^{14}C data are expressed as the F^{14}C fraction modern nomenclature developed for post-bomb data (64). F^{14}C is a concentration unit (^{14}C/C) denoting enrichment or depletion of ^{14}C in relation to oxalic acid standard normalized for isotope fractionation. In addition, data are also reported as decay-corrected Δ^{14}C according to published nomenclature (40). Δ^{14}C was calculated using the equation,

$$\Delta^{14}C = 1000 \times \{F^{14}C \times \exp[\lambda \times (1950 - y)] - 1\} \quad \text{(Eq. 3)}$$

where $\lambda = 1/8267$ year-1 and y is the year of measurement after 1950 A.D.

Determining Year of Birth from 14C Data

The average age at which enamel is formed for each specific tooth has been reported previously and is dependent on the tooth number and is for some teeth different between sexes (17, 39, 44, 65). If the sex of a person is unknown, the average time for enamel formation for males and females is calculated. The ^{14}C concentration measured in the tooth enamel is plotted onto a curve of atmospheric ^{14}C against time to determine the year of enamel synthesis and date of birth of the individual. The time (in years) taken for the enamel to form is subtracted from the year obtained to give an estimated

date of birth. Calibrated ages were obtained by using the CALIbomb Levin data set where the smoothing in years was set at 1.0 and 2 σ error was used. For ^{14}C tooth enamel values that fell between 1955 and 1960, the CALIbomb program was not used. In CALIbomb, these values are predicted using a straight line from prebomb values to 1960. Instead, for ^{14}C levels matching this time period, reference values reported by Hua and Barbetti (66) were used.

2.2 SEX DETERMINATION

DNA was extracted from small fragments of 30 roots of teeth according to a previously described method (67) for bone DNA extraction. In short: the roots were placed in 96 % ethanol for a few minutes and rinsed with 0.5 % Na-hypochlorite, and dried overnight at 56^{o}C in open tubes. The samples were then grinded to a fine powder in liquid N_2 in a Freezer/Mill 6850-115 (SPEX Certiprep, New Jersey). The grinding cycles were the same as for standard bone treatment, but shortened to only half a minute. DNA was extracted from the total amount of tooth powder using one phenol-chlorophorm and one chlorophorm extraction (68), concentrated on Centricon 30 columns (Millipore), purified using the Qiaguick Purification Kit (Qiagen) and eluted in 45 mL buffer.

The DNA concentration was measured with NanoDrop® (Thermo Fischer Scientific, Wilmington, USA). Duplicate aliquots of approximately 1 ng were used for the PCR amplifications according to the Identifiler™ protocol (Applied Biosystems) in a final volume of 10 mL. To verify the results in weak samples these were also amplified with a two-phase PCR protocol of 10 and 20 cycles as previously described (67). The DNA profiles, including amelogenin, the marker for the determination of gender (69), were analyzed by capillary electrophoreses in an ABI3100 and evaluated using GeneMapper ID 3.2 (Applied Biosystems).

2.3 ASPARTIC ACID RACEMIZATION

The aspartic acid racemization analysis was performed according to a previously described protocol (35). Briefly, approximately 1 mm-thick median longitudinal sections produced by cutting the teeth with a low speed cutter (Isomet, 11-1180, Buehler, Chicago, IL). Other components except dentin were carefully removed from the sections, and next, the dentin was rinsed with ultrasonic waves sequentially in 0.2 M HCl, distilled water(three times), ethanol, and ethyl ether for 5 min, respectively.

Then the dentin sections were pulverized in an agate mortar. 10 mg of the powder was used for determination of the racemization ratio in the sample. D-Asp and L-Asp were determined by gas chromatography using a glass capillary (GC-17A, Shimadzu, Kyoto, Japan) after hydrolysis and derivatization. A glass capillary, 30 m in length, and 0.3 mm in internal diameter was used as a column, and coated with Chirasil-Val (GL Science, Tokyo, Japan). The concentration of D-Asp was compared to the content of L-Asp, and the racemization ratio was expressed as ln[(1 + D/L)/(1 − D/L)]. Plotting the chronological age on the x axis and the racemization ratio on the y axis, the following equation by linear regression using the least square method was derived,

$$\ln[(1 + \text{D/L})/(1 - \text{D/L})]_t = 2kt + \ln[(1 + \text{D/L})/(1 - \text{D/L})]_{t=0}$$

where ln[(1 + D/L)/(1 − D/L)] represents the log-transformed racemization ratio, t the chronological age, and k the racemization rate constant. To estimate the chronological age, the age was plotted on the y axis and the racemization ratio on the x axis and the following linear regression equation was derived by the least square method.

$$t = \{\ln[(1 + \text{D/L})/(1 - \text{D/L})]_t - \ln[(1 + \text{D/L})/(1 - \text{D/L})]_{t=0}\}/2k \quad \text{(Eq. 2)}$$

The estimated age was obtained by substituting the D/L ratio in this linear regression equation with that of the specimen to be estimated. For a detailed flowchart illustrating the method of racemization analysis see (33) .

3 RESULTS AND DISCUSSION

3.1 PAPER I

Regression analysis of date of birth estimations using bomb-pulse radiocarbon dating of tooth enamel and aspartic acid racemization analysis of crown dentin revealed a strong correlation between the two methods which each showed a correlation to the actual date of birth of R^2=0.996 and R^2=0.923, respectively, see Figure 7. Although both methods are in good agreement with each other, radiocarbon dating offered more precise age estimations than aspartic acid racemization analysis.

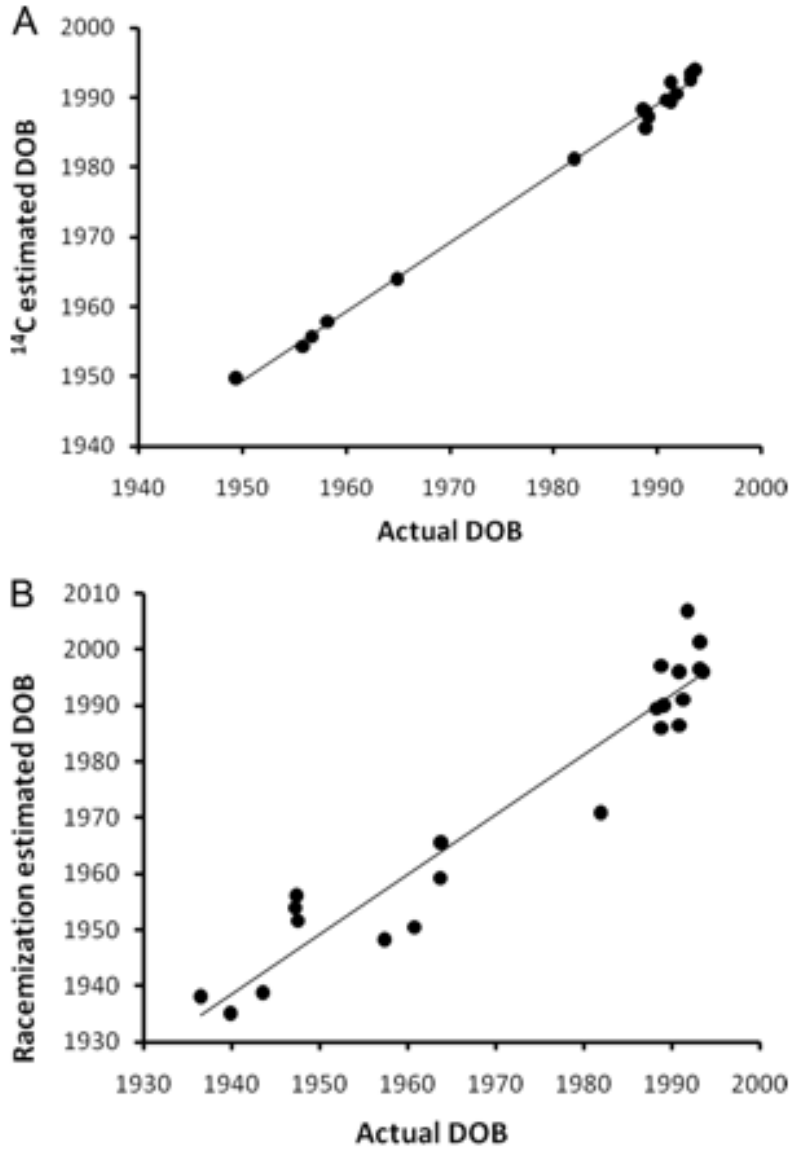

Figure 7. Linear regression plots showing the precision of radiocarbon analysis and aspartic acid racemization analysis of tooth enamel.

Analysis of the whole crushed crown as a more rapid alternative to purified enamel also resulted in a fairly precise date of birth estimation. Crushing the complete tooth crown significantly simplifies the processing needed for radiocarbon analysis and cuts the preprocessing time down to 15 min. Surprisingly good date of birth estimation was also obtained for one whole tooth crown subjected to AMS analysis without any pretreatment, although the number of samples was limited. In this paper, a case report concerning an unknown dead body is also reported. The combined use of radiocarbon analysis and aspartic acid racemization of teeth collected from the victim provided an estimate of the year of birth and year of death of the subject, which assisted the police investigation.

3.2 PAPER II

Teeth formed during the rising part of the bomb curve, between 1955 and 1963, showed an absolute error from the true formation time of 1.9 ± 1.4 years. Despite significant variations in the atmospheric levels recorded during this initial phase, a good correlation was found between the estimated and the actual time of formation (R^2 = 0.751). Teeth formed after1963 (n = 66) showed an absolute error from the true formation time of 1.3 ± 1.0 years. All of these teeth contained bomb pulse derived radiocarbon and showed an excellent correlation with the actual time of enamel formation (R^2 = 0.989). The chance of achieving a close representation of the actual year of birth from a given enamel radiocarbon result along the bomb curve is displayed in Figure 8.

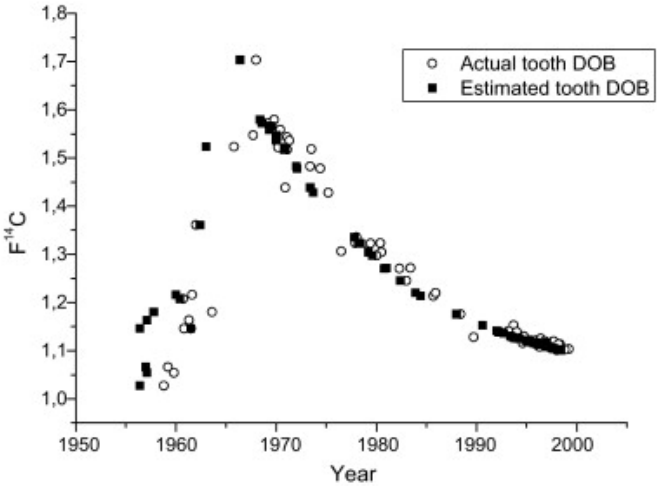

Figure 8. The estimation error of radiocarbon tooth dating along the bomb-curve.

^{14}C dating of teeth obtained from subjects raised on different continents, including South America showed similar precision (Table 2).

Table 2. The precision of the radiocarbon birthdating of teeth from different geographical regions.

Zone	Levin	Zone data	*N*	Countries
SH	1.4 ± 0.5	1.3 ± 0.8	15	Chile and Uruguay
NH Zone 2	1.9 ± 0.9	1.6 ± 1.0	18	Japan, Middle East and Morocco
NH Zone 1	1.0 ± 0.6	1.2 ± 0.8	27	Sweden
NH Zone 1	1.5 ± 1.3	1.5 ± 1.2	35	Scandinavian?

The amount of enamel in teeth from these subjects also allowed for determination of ^{13}C levels. There was an obvious difference in these levels between subjects of different origins. The greatest depression (i.e. lowest concentrations) of $\delta^{13}C$ was seen in teeth from Sweden (-14.7 ± 0.4), followed by teeth from Japan (-13.5 ± 1.2), Middle East (-13.7 ± 0.6) and South America (-10.9 ± 0.6). The differences between these groups were significant ($p < 0.01$), except between teeth obtained from Japan and Middle East. It can be appreciated that there was no overlap at all between teeth from Sweden and South America regarding the ^{13}C concentration.

3.3 PAPER III

This study was performed on teeth from subjects raised exclusively in North Americat. For teeth laid down after 1955, a high correlation was found between the ^{14}C levels in enamel and the actual formation time of the tooth enamel with an average absolute error of 1.8 ± 1.3 years, $R^2 = 0.988$. In this study we also analyzed the ^{14}C levels in the roots from 28 teeth collected from 26 individuals. The ^{14}C levels were consistently higher in the root than in the enamel if the enamel was formed during the rising part of the curve, and lower in the root if the enamel was formed during the falling part of the curve. In all pre-bomb cases, i.e. when the enamel was completely formed before the bomb pulse, post-bomb values were found in their corresponding roots, indicating that a turnover in some component of the root continues even at old age.
The ^{13}C levels in the tooth enamel were analyzed with isotopic ratio masspectrometry. These teeth extracted from subjects raised in North America showed higher enamel ^{13}C

values as compared to levels previously seen in teeth collected from other continents, actually even higher than the levels in teeth from South America (50). The levels were somewhat lower in teeth from California, British Columbia and from Connecticut as compared to teeth from Texas, and enamel from teeth from Mexico showed very high levels, actually higher than any previously analyzed teeth. We also analyzed ^{13}C in tooth roots from teeth in which ^{13}C enamel levels also were measured. The ^{13}C levels in the roots were generally higher than in enamel. Further, the ^{13}C levels in tooth roots were higher in teeth from Mexico than in roots from teeth from United States and Canada, but showed a higher variation, making it more difficult to separate Mexican subjects from persons raised in United States and Canada using roots only.

The ^{18}O levels were in general lower in tooth roots from the northwestern region of North America as compared to those in roots collected from Texas. Mexican teeth showed slightly lower values than teeth from Texas, and were similar to the average for all teeth from United States and Canada.

A small part of the root of teeth was used for DNA analysis. A full profile was obtained for 22 of the teeth, and in all cases, the sex could be determined using two different markers. From four cases, two teeth were analyzed, and the results were identical for the different teeth from the same individual.

3.4 DISCUSSION

The resistance of teeth to environmental influence makes them particularly valuable in the forensic setting. Teeth can remain intact for an appreciable time and allow for an odontologic identification of even very old skeletons provided ante-mortem data are available. Furthermore, no exchange of carbon will take place in the enamel of the mature tooth during life and generally not after death, making mature permanent teeth particularly suitable for radiocarbon analysis.

The studies performed show that both aspartic acid racemization and AMS radiocarbon analyses can be performed on a single tooth and that the combined analysis can provide information about the year of birth and year of death of an individual. Both methods was found to correlate well. The application of radiocarbon analysis of enamel for year of birth determination has previously been reported to show a high precision with an average estimation error of ±1.6 years (39). In the present studies, the high precision was confirmed, when analyzing a large number of teeth from different geographical regions. The aspartic acid racemization analysis of dentin also allowed for a fair

prediction of the age of the person with a precision comparable to that reported previously (for a review, see (34)).

Both methods have strengths and limitations. The radiocarbon birth dating method can tell the birth date of the person regardless of the time of death. However, the time window for this analysis is limited to subjects born after the early 1940s. Hence, one obvious advantage of aspartic acid racemization analysis is that it is independent of the bomb spike and hence can be used for age determination of subjects born long before the beginning of above ground nuclear weapon testing. Several factors, however, will affect the precision of this method. Because the racemization process is basically a function of temperature and time, teeth are exposed to different ambient temperatures depending on their position in the oral cavity. Ohtani *et al.* (70) demonstrated that racemization rates differ between the same tooth in middle-aged versus elderly individuals. The types of teeth the provides the best precision with racemization analysis are single rooted teeth such as mandibular incisors or mandibular premolars (33, 70). Hence, when these teeth are not available, this method becomes less accurate. Another limitation is that the racemization process does continue after death, although at a slow pace (28), implying that the estimation of age at death might not be accurate regarding bodies that died many decades ago. For the radiocarbon analysis, the standard extraction procedure was simplified as compared to the previously reported protocol (39) by using an electric odontological drill to remove dentin. Hence, enamel could typically be isolated within 4 days. The freezing mill used to crush teeth may turn out to be an even more attractive alternative to more rapidly obtain an estimate of the date of birth, since it seemingly provides similar precision. It should be pointed out that age determination is particularly important to limit the search for possible missing person matches and that this information may be badly needed in the early phase of an investigation whether it concerns a crime or a mass disaster. Because radiocarbon analysis also gave good precision with the crushed teeth where the whole crown was ground down, we conclude that the exchange of carbon between the dentin within the crown and the environment typically is negligible. This has also been shown by aspartic acid racemization of crown dentin (71).

The power of the bomb pulse radiocarbon method is likely to only improve in the coming years. Three reasons for this are as follows. 1) tooth status in the population has improved substantially, and a dead vicitm is more likely to have teeth left for analysis in the coming decades than today, 2) the population exposed to bomb spike radiocarbon rapidly increases. This means that a growing number of unknown dead bodies are expected to have remaining teeth containing bomb spike-derived ^{14}C, and 3) the

precision of AMS analysis improving, implying that a lesser amount of intact enamel will be necessary for analysis. Actually, radiocarbon may also be analyzed with a even more sensitive method, so-called intracavity optogalvanic spectroscopy, which can allow for analysis of sub-microgram samples of carbon(72). This means that even teeth with minimal remnants of enamel may be dated in the future.

Two main concerns can be raised about the precision of the bomb-pulse radiocarbon method. First, since the initial distribution of bomb pulse derived radiocarbon around the world was somewhat uneven (40, 66), with a lag in the southern hemisphere, the global applicability of this birthdating method by analysis of tooth enamel may be questioned. Second, the standardized measurements of radiocarbon levels in the atmosphere did not start until 1959 (66, 73), so before that, the levels are based on various other kinds of measurements, implying that the actual concentrations at different geographical regions are uncertain. In addition, the continuous above-ground detonations of new nuclear test bombs before 1963 implied that the levels varied from year to year, hence the dating of biological material that incorporated atmospheric radiocarbon between 1955 and 1963 is expected to be less accurate. In paper II, these two questions were addressed. Teeth were collected by dentists in Scandinavia, Japan, Iraq, Kuwait, Marocco, Uruguay and Chile and subjected to analysis of radiocarbon in enamel. The precision in dating turned out not to differ much between subjects from these different geographical regions, and importantly, there was no significant systematic underestimation of age of teeth from subjects raised in South America. We also evaluated the precision of the radiocarbon dating for teeth formed during the rising and falling part of the bomb curve. We found that although the teeth formed before 1963 provided less accurate age estimation, but still the average absolute error was good; 1.9 ± 1.4 years. Hence, it can be concluded that age determination using bomb-pulse radiocarbon in tooth enamel is a globally applicable method, with a precision all along the bomb-curve that surpasses any other method for age estimation. It was also found that the comparison with the global average (73) gave similar estimation as did the use of regional reference data (http://intcal.qub.ac.uk/CALIBomb/frameset.html) This means that the dating of a person with unknown origin will seemingly be equally precise regardless of where the person actually grew up.

In contrast, the stable isotopes ^{13}C and ^{18}O display geographical differences. In paper II, ^{13}C was measured in teeth from subjects raised on several different continents, and in paper III, ^{13}C and ^{18}O was measured in teeth from Mexico, United States and Canada. The differences in ^{13}C concentrations were amazing, when comparing e.g. Scandinavian and South American subjects, but actually, even in such a limited

geographical area as North America, Mexicans could easily be separated from subjects raised in the United States or Canada. These results compare well with those recently reported regarding stable isotope analysis of hair samples from different countries around the world (49). However, the ^{18}O levels were more difficult to interpret, partly because this isotope shows a more complex distribution pattern. Having stated that, analysis of a set of several different stable isotopes, it is likely that a better idea of the origin of a subject can be obtained. The teeth from North America were also subjected to ^{14}C analysis and showed an average absolute error of 1.8 ± 1.3 year. This precision is similar to that of previous studies on teeth obtained from other countries around the world (37, 39, 50, 65, 74). Further, several teeth from the same individual was analyzed and provided similar estimates of the persons year of birth. The number of teeth was however not sufficient to tell which type of tooth that shows the best precision, but seemingly, radiocarbon of any type of tooth will provide a better estimate of the year of birth of a person than other alternative analytical methods. Finally, the estimates of year of birth using Nolla´s reference data on tooth formation was compared to the estimates using radiocarbon as such on all teeth analyzed in these papers, and in a previous study (39). The estimates were similar, but there was a tendency to overestimate age when using Nollas´s reference times. This might be explained by a difference between the average time for incorporation of carbon into the tooth and the average density as viewed on a dental radiograph. Only 0.5 % of the tooth enamel is carbon, and most of the radio-opacity will depend on the hydroxyapatite content, a cristalline structure devoid of carbon. Having stated that, the time interval between the incorporation of atmospheric ^{14}C levels into plants and the incorporation into tooth enamel will introduce both a systematic and a random error, if the crude values should be used to estimate average ^{14}C incorporation times in enamel. However, the tabulation in paper III of the actual differences found between tooth age as assessed by ^{14}C *per se* and the true date of birth of the person provides a simple guide to the interpretation of ^{14}C values obtained, although for some tooth types, more data are warranted to improve the precision of the interval.

3.5 CONCLUSIONS

These studies have shown that analysis of ^{14}C in teeth can provide a very precise birthdating of unknown individuals, regardless of geographical origin in subjects born in the 1940s and later. The analysis of either two teeth with different formation times,

or of the enamel and the root of the same tooth could provide a clear indication of their formation times and improve the interpretation of the radiocarbon analysis results. The levels of ^{13}C and ^{18}O in tooth roots provide information about diet and/or geographical origin with significant differences between populations in discrete geographical regions. The feasibility of aspartic acid racemization, previously reported, and used for determination of age at death, was confirmed by application on teeth with known extraction dates and formation times and was found to be useful to tell the date of death if the date of birth could be estimated by radiocarbon analysis. Sex determination using DNA analysis of dentin required only small samples and could be performed along with a full DNA profile in most samples. Analysis of stable isotopes in enamel and roots showed that most subjects apparently consume food from similar primary dietary sources from infancy throughout childhood.

Finally, it should be pointed out that the studies performed provides clues to the identity, in order to limit the search for possible matches, and that these methods (apart from the DNA profiling) do not replace regular identification procedures. However, whenever there are no clues as to the identity of a dead person (not necessarily a homicide victim or a mas disaster victim), the proposed strategy may be an asset to the death investigation in the early phase.

3.6 FUTURE DIRECTIONS

Among unidentified victims, some are victims of fire, and high temperature may affect the proportion of ^{14}C in the teeth. In these studies, such teeth have not been examined. Although there are no reports indicating that the ^{14}C levels should be affected differently from the ^{12}C levels during heat exposure, it would be interesting to investigate the applicability of this methodology on tooth enamel and tooth roots from fire victims to facilitate the identification. Analysis of additional stable isotopes in teeth are expected to provide further clues to the place where a unknown victim was raised. The reference table regarding average ^{14}C incorporation time in the enamel is a shortcut to determine the date of birth, but more teeth of certain types of both sexes are warranted to produce a reference guide that eliminates the detour via dental radiographical data. The combination of ^{14}C levels in tooth enamel and aspartic acid racemization of dentin may provide both year of birth and year of death, however ^{14}C analysis of hair (when available), which is expected to contain contemporary levels might be a convenient strategy to replace the aspartic acid racemization analysis and hence expedite the investigation.

Finally, stable isotope analysis of multiple samples, such as enamel, tooth root, bone, hair (and soft tissues when available) would be most interesting to perform to pinpoint the possible changes in diet, habits and geographical movements over time of unknown victims. The signatures of radiocarbon and stable isotopes in the different tissues of the body are invisible to the naked eye, but may prove to have more to tell when further explored.

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